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TECHNICAL MANUSCRIPT 536

CONTROL OF VIRAL RNA SYNTHESIS
IN EASTERN EQUINE ENCEPHALITIS VIRUS

Eugene Zebovitz
Arthur Brown

JUNE 1969

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TECHNICAL MANUSCRIPT 536

CONTROL OF VIRAL RNA SYNTHESIS IN EASTERN EQUINE ENCEPHALITIS VIRUS

Eugene Zebovitz

Arthur Brown

Virus & Rickettsia Division
BIOLOGICAL SCIENCES LABORATORIES

Project 1B061102B7 1A

June 1969

ABSTRACT

The pattern of viral RNA synthesis and growth of a temperature-sensitive mutant (Ets-4) of eastern equine encephalitis (EEE) virus was compared with that of the parent. Growth of Ets-4 was moderately inhibited at 30 and 37 C compared with that of EEE, and was strongly inhibited at 42 C. In Ets-4, similar patterns of inhibitions were observed in the production of complement-fixing antigen and hemagglutinin (HA), which indicated that Ets-4 was defective in viral protein synthesis.

The viral RNA synthesis induced by both viruses in infected cells was studied extensively. The mutant produced three times as much total viral RNA as EEE virus at 37 C, but less infectious RNA (IRNA). Comparison of the specific infectivity (IRNA/total viral RNA) of the two viruses showed that of Ets-4 to be 0.1 that of the parent. RNA from each of the viruses was analyzed more completely with sucrose gradient centrifugation. Ets-4 at 37 C produced greater amounts of a noninfectious 20S species (double-stranded forms, ribonuclease-resistant) and a poorly infectious 27S form (single-stranded, ribonuclease-sensitive) of viral RNA. Production of single-stranded infectious species, 45S, of viral RNA by Ets-4 was less than that by EEE virus, but the total uptake of radioactive uridine was higher, suggesting that the 45S species of RNA synthesized in Ets-4 - infected cells was biologically defective.

On the basis of the facts noted above, it appeared that viral RNA synthesis in Ets-4 - infected cells was out of control. Control appeared to be restored to the levels and pattern of the parent by superinfection with the parent EEE virus or VEE virus but not with others.

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I. INTRODUCTION*

A number of reports have appeared recently that indicate that after infection of cells by group A arboviruses, a number of different classes or species of viral RNA are formed.¹⁻⁴ Generally the classes or species of RNA have been divided on the basis of their sedimentation coefficients calculated from data obtained after sucrose gradient centrifugation. Thus far, available data indicate that one or more double-stranded forms (16S to 20S)** of viral RNA are formed initially in the infected cell and that almost simultaneously or soon thereafter a single-stranded, relatively non-infectious "interjacent"*** 27S RNA is found. This is followed by the appearance of a single-stranded infectious 45S species associated with the mature virus particle. There are a few variations on this theme, and the precise nature, function, and interrelationships of each species are not completely known. Furthermore, little information is available on control of viral RNA synthesis. This paper is a report on an abnormal pattern of viral RNA synthesis induced by a temperature-sensitive mutant (Ets-4) of eastern equine encephalitis (EEE) virus at 37 C. Compared with its parent, the mutant appears to have lost control of the synthesis of its RNA. Control apparently can be restored to equal that of the parent by superinfection with the parent or VEE virus, but not by certain other viruses.

* This report should not be used as a literature citation in material to be published in the open literature. Readers interested in referencing the information contained herein should contact the senior author to ascertain when and where it may appear in citable form.

** No distinction is made here between the replicative form (RF) and the replicative intermediate (RI).⁵ The former is double-stranded RNA and the latter may be a double-stranded RNA with nascent single strands attached. In this paper, both forms will be described together as 20S, since they were observed as one peak after centrifugation in a sucrose gradient (20S-16S), although on occasion one could distinguish two peaks.

*** The "interjacent" RNA¹ has been calculated to have a value of 27S, and the infectious RNA, 45S. In the papers of other arbovirus workers, an "interjacent" RNA has been described as 26S to 28S, and the highly infectious RNA as 40S to 47S.

II. MATERIALS AND METHODS

Cell cultures, virus infection and growth, plaquing, etc., have been described in detail previously for certain group A arboviruses and chick embryo (CE) cells.^{6,7}

A. VIRUS STRAINS

The parent virus was the Louisiana strain of EEE virus, whose properties have been described by Brown⁸ and Zebrovitz and Brown.⁷ CE cell culture seeds usually contained between 1×10^9 and 8×10^9 plaque-forming units (pfu) per ml.

The temperature-sensitive mutant Ets-4 was obtained after treating with nitrous acid the infectious RNA that was isolated from the parent virus⁸ to produce a 99% decrease in titer. A seed was then made at an incubation temperature of 30 C and subsequently plaqued at 30 C. Plaques were picked at random and replaqué in duplicate sets of plates, one of which was incubated at 30 C and the other at 42 C. Those isolates that formed plaques at 30 C but not at 42 C were further purified three times by replaqué; seeds were then made in CE cells at 30 C.

Such seeds usually contained between 1×10^9 and 8×10^9 pfu/ml and those that had a reversion frequency (ratio of number of plaques formed at 42 C to number of plaques formed at 30 C) of less than 10^{-4} were selected as candidates for temperature-sensitive mutants. Only after it was demonstrated that multiplication of the candidate virus was inhibited significantly in liquid monolayer cultures were the candidates accepted as valid mutants, because occasional clones were encountered that could not plaque at 42 C under agar but were only slightly inhibited in growth at 42 C in liquid monolayer cultures. Ets-4 was one of the mutants selected. Its growth was markedly inhibited at 42 C, but it was able to induce the synthesis of substantial quantities of viral RNA at this temperature (see below); it was therefore designated as RNA+,⁴ in contrast to those that failed to synthesize RNA at 42 C (RNA-).

In certain experiments, cultures infected with Ets-4 were superinfected with a number of different viruses. These included the following viruses from our laboratory CE cell culture stocks: Venezuelan equine encephalitis (VEE) (Trinidad), Sindbis (S) (AR339), Semliki Forest (SF), vesicular stomatitis virus (VSV) (New Jersey), and Newcastle disease virus (NDV).

B. VIRAL RNA PROCEDURES

1. Uridine Uptake by Infected Cells

Viral RNA synthesis was followed by the incorporation of radio-active uridine in infected CE cells in the presence of actinomycin D.

CE cells were infected as described above, overlaid with 2 ml of a minimal medium* containing actinomycin D, 1 µg/ml; and C¹⁴-uridine, 0.025 µc/ml. Insulin at 0.27 unit/ml was also added to the medium to enhance the incorporation of radioactive uridine.¹⁰ Cultures were incubated at the desired temperature in an incubator supplied with a mixture of 5% CO₂ and 95% air. At 2-hour intervals, duplicate petri plates were taken from the incubator, the medium was removed, and 2 ml of 10% trichloroacetic acid (TCA) were added. The cells were scraped off the plates with a rubber policeman and put into a centrifuge tube, washed twice with 10% TCA by centrifugation, and trapped on membrane filters. The radioactivity was subsequently determined with a Packard liquid scintillation spectrometer.

2. Extraction of Viral RNA

Virus-infected cells were overlaid with 2 ml of the minimal medium just described that contained either C¹⁴-uridine (0.025 µc/ml) or H³-uridine (1 µc/ml). The cultures were held at 37 C for 6 hours. The cells were scraped off the plates and suspended in 0.02 M phosphate and 0.0001 M ethylenediaminetetraacetic acid (EDTA), pH 7.4. Viral RNA was extracted from the cells with hot (60 C) water-saturated phenol and 0.1% sodium dodecylsulfate (SDS). Two phenol extractions were performed and the aqueous phase was precipitated with three volumes of ethyl alcohol containing 2% potassium acetate. The RNA was stored in alcohol at -70 C until required. The RNA precipitate was removed from the alcohol by centrifugation and dissolved in 2 ml of phosphate-buffered saline (PBS), pH 7.4.

3. Assay of Infectious RNA (IRNA)

Viral RNA extracted as described above was assayed on CE cell monolayers made hypertonic by washing once each with PBS, 0.5 M NaCl, and then 1.0 M NaCl in 0.1 M Tris-HCl buffer, pH 8.2.¹¹ RNA dilutions were made in 1.0 M NaCl Tris-HCl buffer blanks. A 0.1-ml sample of the diluted RNA was put on the hypertonic monolayers and allowed to fix for 15 minutes, then the inoculum was removed and overlaid with lactalbumin hydrolysate agar. Cultures were incubated for 2 days at 37 C and stained at that time with neutral red; plaques were counted a few hours later.

4. Sucrose Density Gradient Analysis

Two-ml samples of viral RNA in PBS were layered on a 28-ml sucrose gradient (15 to 35%) in reticulocyte standard buffer (0.01 M Tris-HCl, pH 7.4; 0.01 M KCl; 0.0015 M MgCl₂)¹² containing 2 µg polyvinylsulfate per ml and 0.15 M NaCl, and centrifuged at 22,000 rpm in an SW-25 rotor of the model L2 Spinco ultracentrifuge for 18 hours. Ten-drop fractions were collected from the bottom of the tube. Each fraction was analyzed for optical density at 260 mµ to determine the location of the 28S and 18S ribosomal RNA peaks. Radioactive measurements were made after the viral

* Hanks balanced salts solution, cystine, 75 mg/liter; histidine, 60 mg/liter; sodium bicarbonate, 0.14%.⁹

RNA was precipitated with 10% TCA in the presence of 100 µg of yeast RNA per ml as carrier. The precipitate was collected on membrane filters and the radioactivity was determined with a liquid scintillation counter.

When it was desired to determine the IRNA content of the fractions, 0.1 ml of each fraction was removed immediately after collection and the titer was determined as described earlier for assay of IRNA.

Ribonuclease resistance of the viral RNA in the sucrose gradient fractions was determined with 1-ml portions of each fraction, which were diluted with sterile distilled water to give a final volume of 2 ml. One µg of ribonuclease was added to each sample, which was incubated at 37 C for 30 minutes. The samples were then processed for radioactivity as described earlier.

C. TESTS FOR COMPLEMENT-FIXING (CF) ANTIGEN AND HEMAGGLUTININ (HA)

CF antigen in infected cells was released by scraping the cells off the plates into PBS, pooling them in a test tube, and disrupting them with a Branson sonic oscillator. The cell debris was removed by centrifugation and a CF test was performed with the cell extract. The CF test was performed according to the standardized diagnostic CF method of the U.S. Public Health Service Monograph 74.¹³ The antiserum was obtained from guinea pigs immunized with infectious EEE virus that was purified by centrifugation in a sucrose gradient. This antiserum did not react with host cell components and specifically detected the presence of virus antigen in infected cell extracts. The hemagglutinin (HA) test¹⁴ was performed with serum-free tissue culture fluids (minimal medium)⁹ derived from infected CE cell cultures. Actinomycin D was obtained as a gift from Merck, Sharpe and Dohme.

III. RESULTS

Figure 1 shows the growth response of Ets-4 compared with its parent at three incubation temperatures. EEE virus has the unusual capacity to grow in CE cells at very high temperatures.⁷ The growth response of this virus differed at each temperature, but the final titers achieved at the end of 12 hours were approximately the same. In contrast, Ets-4 growth was more strongly influenced by temperature. The growth response of this mutant never equalled that of its parent virus at 30 C and 37 C; the titer of Ets-4 at 12 hours was generally 0.5 to 1.0 log₁₀ units below that of EEE virus at these temperatures. At 42 C, Ets-4 was strongly inhibited. These data show that Ets-4 is temperature-sensitive and generally exhibits a poorer growth response compared with its parent virus.

The next few experiments were designed to obtain more information on the temperature sensitivity of Ets-4 by comparing the viral protein and viral RNA synthesis of this virus with its parent. In the first group of experiments the production of virus-specific CF antigen and HA were used as indicators of viral protein synthesis. Table 1 shows the results of a typical experiment: the production of CF antigen and HA was reduced in Ets-4 - infected cells compared with EEE-infected at all temperatures, except that inhibition of HA production was more pronounced than CF production. Thus, Ets-4 is apparently defective in viral protein synthesis.

In light of these results, it was next of interest to compare the viral RNA synthesis of the mutant with that of the parent strain. Figure 2 shows the rate of incorporation of C¹⁴-uridine by CE cells infected with EEE and Ets-4 at various temperatures in the presence of actinomycin D. The results indicate that Ets-4, which induced lower infectious titers of mature virus and produced less viral protein than its parent, was nevertheless able to synthesize considerably more viral RNA at 37 C and 30 C and an appreciable amount at 42 C.

Because the levels of viral RNA synthesis induced by Ets-4 were reproducibly highest and occurred earliest at 37 C while virus growth was still inhibited compared with that of the parent, most of the remaining experiments were carried out at this temperature.

The next experiment was designed to compare the total viral RNA (as measured by incorporation of radioactive uridine) and IRNA synthesis for both mutant and parent viruses. Cells infected with Ets-4 or EEE were tested for C¹⁴-uridine uptake in the presence of actinomycin D. The viral RNA was extracted with hot phenol and SDS and assayed for IRNA on CE cell monolayers.

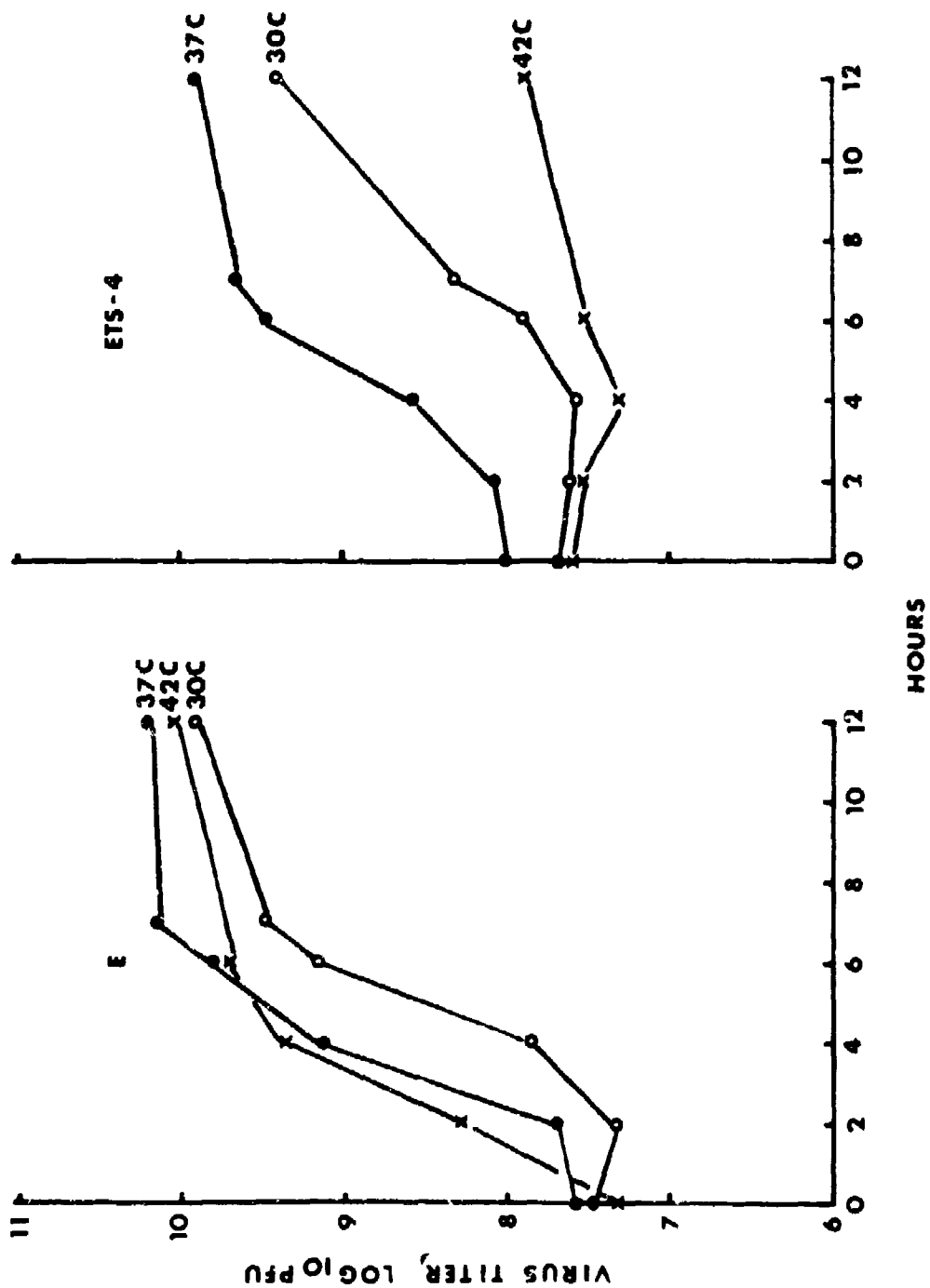


FIGURE 1. Effect of Temperature Upon the Growth Response of EEF and Ets-4 Viruses. \circ — \circ 30°C; \bullet — \bullet 37°C; \times — \times 42°C

TABLE 1. PRODUCTION OF CF ANTIGEN AND HA BY EEE
AND Ets-4 VIRUSES AT INDICATED TEMPERATURES

Time, hour	EEE			Ets-4		
	30 C	37 C	42 C	30 C	37 C	42 C
Complement Fixation						
0	0	0	0	0	0	0
2	0	0	0	0	0	0
4	0	4	4	0	2	2
6	0	32	16	0	8	2
8	4	32	16	2	16	2
10	16	64	8	8	32	4
24	128	32	8	64	8	0
Hemagglutination						
0	0	0	0	0	0	0
2	0	0	0	0	0	0
4	0	64	128	0	32	0
6	16	512	512	0	64	4
10	128	1024	1024	16	128	4
24	1024	4096	512	128	128	0

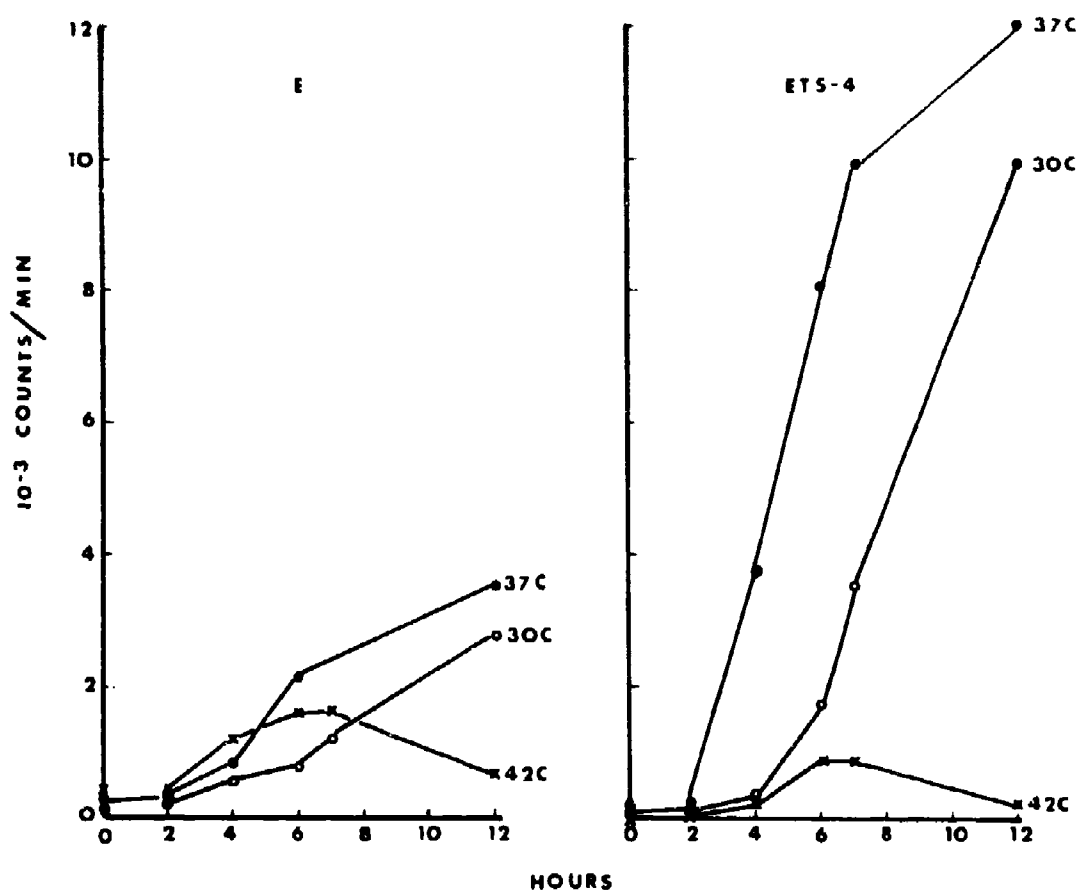


FIGURE 2. ^{14}C -uridine Incorporation by CE Cells Infected by EEE and Ets-4 Viruses at Different Temperatures. \circ — \circ 30°C; \bullet — \bullet 37°C; \times — \times 42°C

Table 2 shows the results of this experiment. The IRNA titer of Ets-4 was only about one-third that of EEE, but the radioactivity incorporated into infected cells by Ets-4 was more than three times higher than that of EEE. The increased incorporation of radioactive uridine in all the experiments described in this paper ranged between two and five times that of EEE, with most values showing a threefold increase. To permit meaningful comparison of the data obtained from both viruses, the data in the fourth column of Table 2 were expressed as specific infectivity, that is, as the ratio of pfu of IRNA to total viral RNA as measured by cpm of C^{14} -uridine incorporated. The values obtained for each virus were in turn expressed as a ratio of the specific infectivity of Ets-4 to that of EEE virus (right column). The results show that Ets-4 had 0.1 the value of EEE. These data indicate that the increased viral RNA synthesis of Ets-4 did not result in a corresponding increased amount of IRNA, but that, in fact, most of the viral RNA synthesis resulted in a poor quality of nucleic acid products as far as infectivity was concerned.

TABLE 2. RELATIONSHIP OF C^{14} -URIDINE UPTAKE
BY INFECTED CELLS TO IRNA
FORMED BY EEE AND Ets-4 VIRUSES

Virus	C^{14} -uridine IRNA. ^{a/} pfu	Uptake, cpm	Specific Infectivity, pfu/cpm	Ratio ^{b/}
EEE	3.1×10^6	38,395	81	-
Ets-4	9.0×10^5	116,590	8	0.1

a. IRNA extracted by hot phenol SDS + C^{14} -uridine uptake determined for approximately 1.0×10^8 CE cells.

b. Ratio = $\frac{\text{Specific infectivity of Ets-4}}{\text{Specific infectivity of EEE}}$

In an effort to analyze further the nature of the viral RNA formed by EEE and Ets-4, CE cells infected with Ets-4 or EEE were incubated for 6 hours at 37 C in the presence of C^{14} -labeled uridine and actinomycin D. The RNA was extracted from the infected cells, dissolved in PBS, and fractionated by sucrose gradient centrifugation. The results in Figure 3 show the sedimentation patterns of RNA from Ets-4 and EEE viruses. The position of ribosomal RNA peaks from chick embryo cells was determined by optical density measurements. Ribosomal RNA peaks for 28S and 18S are indicated by arrows on this curve and were used as reference points to calculate the sedimentation coefficient of the radioactive viral RNA peaks. Three major viral RNA peaks were observed for both viruses. The peak that is characterized by a value of 45S is the IRNA that is incorporated into the mature virus particle. About 98% of the infectivity found in viral RNA resides in this RNA species. Note that the total amount of 45S RNA as measured by radioactivity was much greater with Ets-4 than with EEE, yet the infectivity of this fraction for Ets-4 was lower.

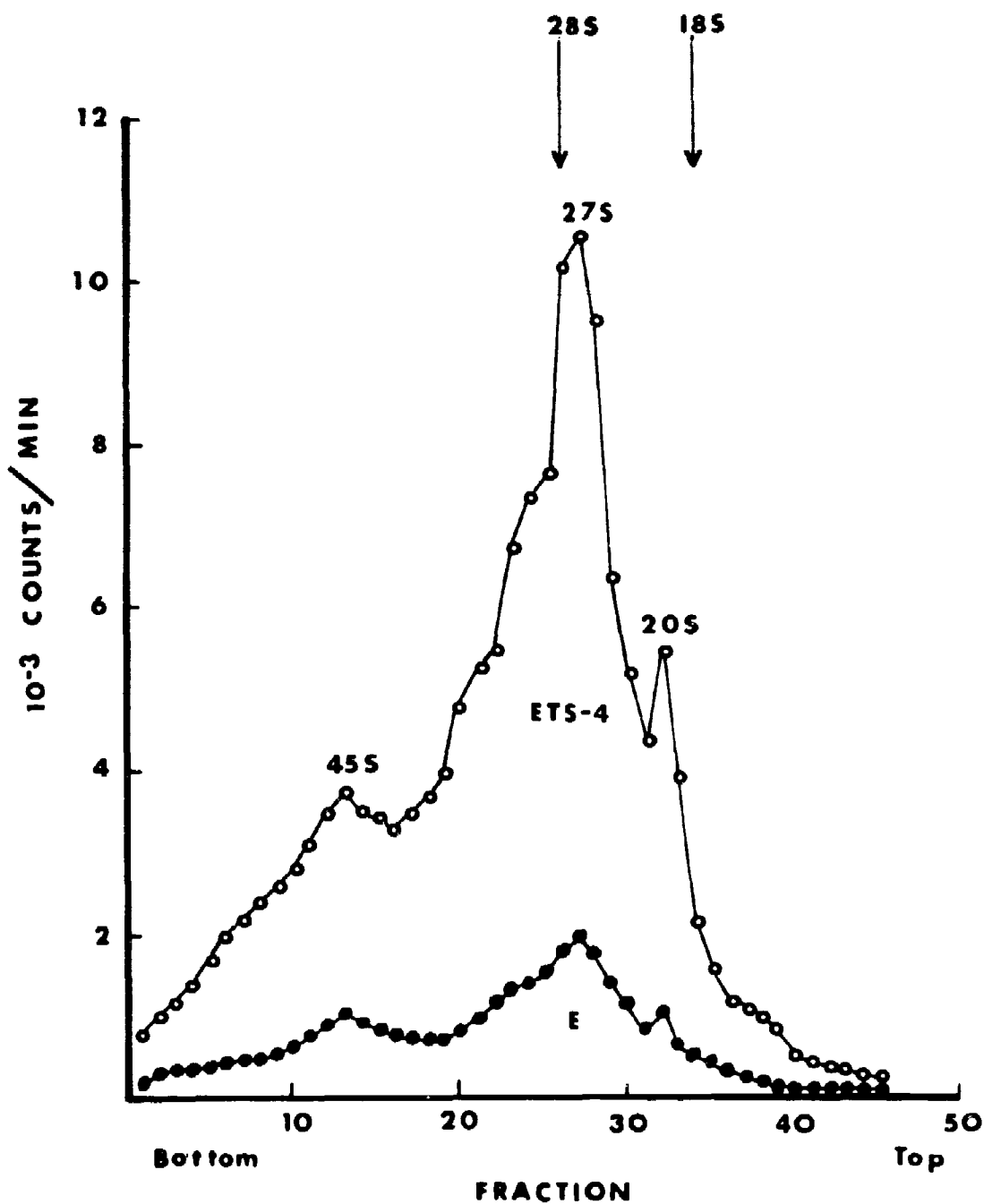


FIGURE 3. Sucrose Gradient Sedimentation of Viral RNA Extracted from Cells Infected by EEE and Ets-4 Viruses and Grown at 37 C for 6 Hours. \circ — \circ Ets-4; \bullet — \bullet EEE

To compare more precisely the relative infectivity of the 45S RNA of Ets-4 with that of EEE virus, the following experiment was performed. Viral RNA was fractionated on sucrose gradients and the infectivity and the radioactivity associated with the 45S RNA were determined and a specific infectivity value was calculated (Table 3). On a specific infectivity basis, the 45S RNA of Ets-4 had approximately 10% the infectivity of EEE virus; however, this value varied among experiments. In some runs, Ets-4 had as high as 30% the specific infectivity of EEE. In all experiments, Ets-4 produced an RNA that was less infectious than that of EEE virus.

TABLE 3. SPECIFIC INFECTIVITY OF THE 45S RNA OF EEE AND Ets-4 VIRUSES

Virus	cpm ^a /	Infectivity, ^b / pfu	Specific Infectivity, pfu/cpm	Ratio ^c /
EEE	1330	1.1×10^9	8.4×10^5	-
Ets-4	2746	2.8×10^8	1.0×10^5	0.12

a. Radioactive count associated with 45S peak fraction in 15 to 35% sucrose gradients.

b. Infectivity of 45S peak fraction.

c. Ratio = $\frac{\text{Specific infectivity of 45S RNA of Ets-4 virus}}{\text{Specific infectivity of 45S RNA of EEE virus}}$

Two other species of RNA were also formed by both viruses. One species sediments at 27S. This RNA had little associated infectivity, and may be a transitional form of RNA that leads to the fully infectious 45S RNA.¹⁶ The third species of viral RNA formed is probably double-stranded; it has a sedimentation value of 20S and is relatively ribonuclease-resistant. In our experiments, it was resistant to 1 μ g of pancreatic ribonuclease per ml.

It appears that Ets-4 induced the synthesis of the same types of viral RNA as did EEE virus, but in much larger amounts, which, it is emphasized again, did not result in correspondingly greater amounts of either IRNA or infectious virus particles.

As a working hypothesis, one may consider the unusual pattern of Ets-4 RNA synthesis as indicating a loss of control by the mutant of its own RNA synthesis that results in the formation of large amounts of poorly infectious RNA. The parent virus presumably has a control mechanism that limits the production of viral RNA to normal levels. One could anticipate, therefore, that it might be possible to restore control of RNA synthesis to normal levels by superinfection of these cells with the parent EEE virus. The latter may provide the necessary control mechanism (e.g., a repressor) to

limit or repress Ets-4 RNA synthesis to normal amounts. Presumably, the repressor is absent or defective in Ets-4 - infected cells. The results of an experiment to test these ideas are shown in Figure 4.

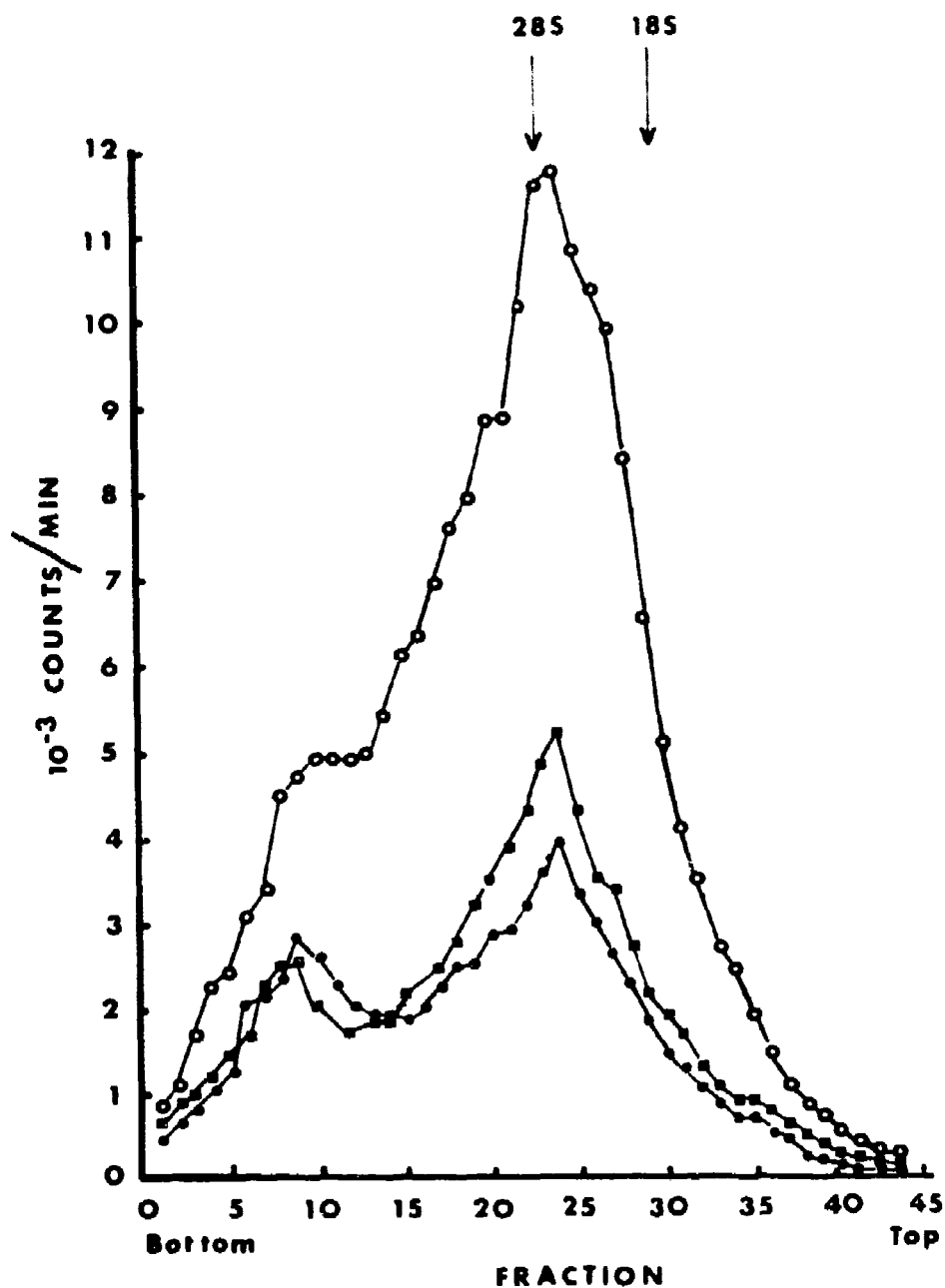


FIGURE 4. Effect of Double Infection of CE Cells with EEE and Ets-4 Viruses Upon the Viral RNA Sedimentation in Sucrose Gradients.
 ○—○ Ets-4 Virus; ●—● EEE Virus; x—x Ets-4 + EEE Viruses

This figure shows the sedimentation pattern of RNA extracted from CE cells infected at the same time and with approximately the same multiplicity of EEE and Ets-4 viruses. The input multiplicity of infection of this experiment was adjusted to 10 pfu/cell. The viruses were allowed to replicate together for 6 hours. The RNA sedimentation pattern of the doubly infected cells remained the same in that the same peaks were still observed, but the amount of viral RNA formed in these cells was reduced to a level that closely approximated that of EEE alone. These data support but do not prove the notion that control was restored. The question arises whether or not the restoration of control is only apparent and whether, instead, the pattern of RNA synthesis in doubly infected cells was due to interference of Ets-4 growth by EEE virus and not directly related to the fundamental regulatory mechanisms of viral RNA synthesis. This alternative explanation is considered unlikely because direct tests for interference in this experiment, and later ones with other group A arboviruses, revealed no difference in the growth curves of Ets-4 or EEE virus in singly or in doubly infected cells; in fact, it was somewhat disappointing to find that the apparent restoration of control did not result in an increase in infectious Ets-4. Furthermore, in an extensive study of interference among group A arboviruses in cells treated with actinomycin D,¹⁶ interference occurred only if one virus had an advantage over the other in time or in multiplicity of infection. EEE virus and Ets-4 were no exception in this regard; thus, interference was apparently not involved in the experiments reported above.

The next experiment was designed to determine the time limit during which superinfection with EEE virus could dominate the pattern of viral RNA synthesis in cells doubly infected with EEE virus and Ets-4. The data in Figure 5 show that superinfection was effective if EEE virus was added up to 1 hour but not at 2 hours after Ets-4. It is possible that at 2 hours of infection the apparent failure to exert control of Ets-4 RNA synthesis by EEE virus might be due to other factors, such as interference by Ets-4 in EEE virus growth that may be manifested only at or after 2 hours.¹⁶ It seems reasonable to conclude, however, that EEE virus can influence Ets-4 RNA synthesis during the first hour, and that an event following infection by EEE virus determines the restoration of control.

Next, we determined the specificity of the requirement of the super-infecting virus for restoring control on the pattern of Ets-4 RNA synthesis. In order to examine a number of viruses, the criterion chosen for comparing viral RNA synthesis in cells singly infected with each virus and doubly infected with Ets-4 was the total RNA synthesis at 10 hours after infection; Ets-4 was the only virus that by itself at 37 C induced very high levels of uridine incorporation. The 10-hour results are representative (Table 4) and show that VEE virus was as effective as EEE in restoring control, but VSV, NDV, and certain other arboviruses were not effective. The reasons for this intermediate degree of specificity are presently unknown, although a number of possible explanations are being investigated.

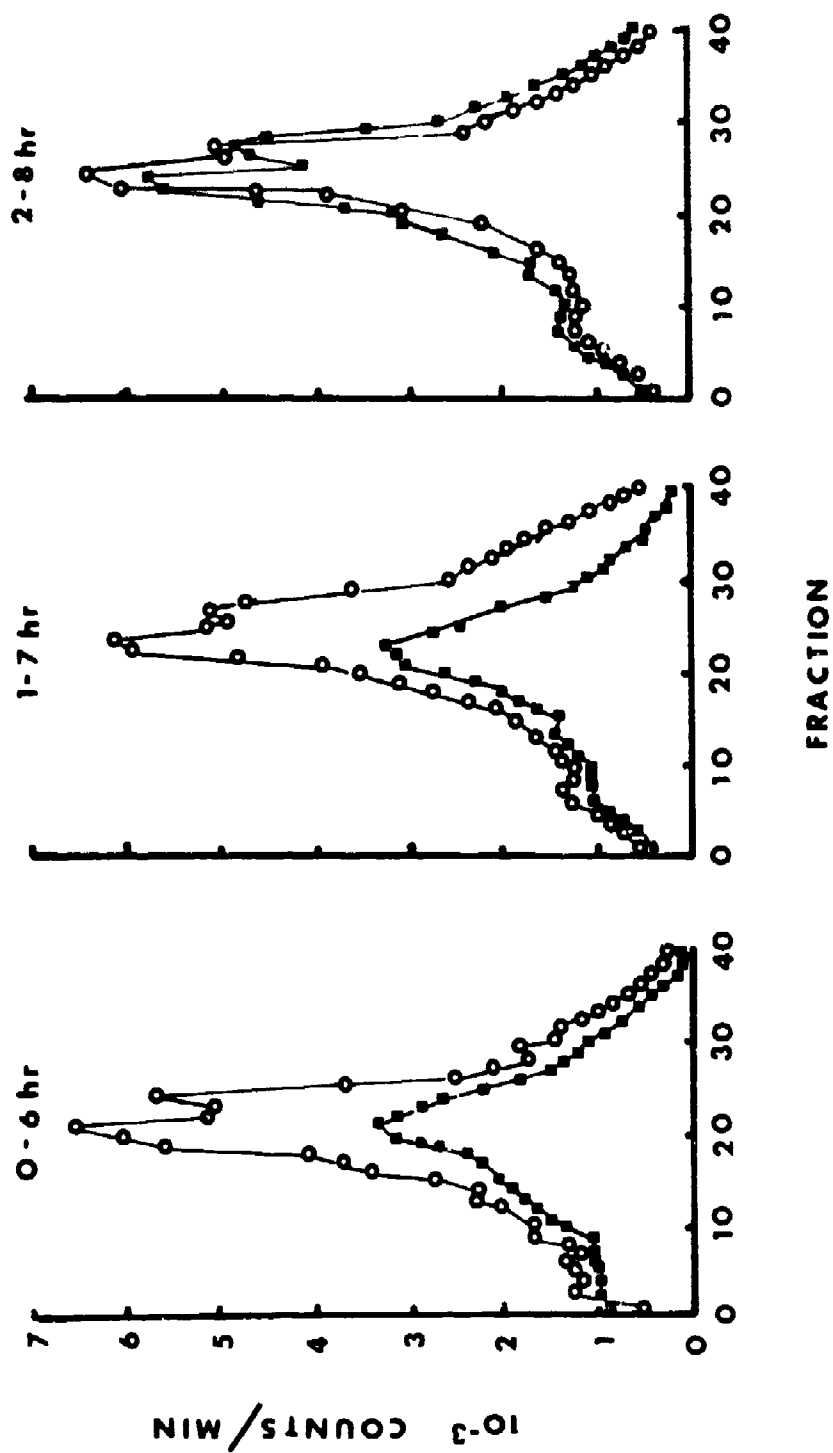


FIGURE 5. Effect of Time of Superinfection with EEE Virus on the Synthesis of Ets-4 Virus RNA. \circ — \circ Ets-4 Virus; \times — \times Ets-4 + EEE Viruses

TABLE 4. INHIBITION OF VIRAL RNA SYNTHESIS IN CELLS
INFECTED SIMULTANEOUSLY WITH OTHER VIRUSES

Virus	% Inhibition in C^{14} -uridine Incorporation
Ets-4 (control)	0 ^a /
EEE	60
VEE	60
Semliki Forest	24
Vesicular stomatitis	22
Newcastle disease	5
Sindbis	2

a. C^{14} -uridine incorporation of Ets-4 - infected
CE cells after 10 hours was approximately 15,000
cpm/ 10^7 cells.

IV. DISCUSSION

The information accumulated indicates that Ets-4 is a temperature-sensitive mutant of EEE that may be classified as RNA+,² and that is defective in viral protein synthesis. What is of greater interest, however, is that at 37 C, where the synthesis of infectious virus and of viral protein are reduced compared with those of EEE virus, Ets-4 induces the synthesis of excessive amounts of viral RNA.¹⁷ The specific infectivity of the RNA (IRNA/total viral RNA) is low. The RNA products consist largely of the species that have relatively poor infectious quality (the 20S and 27S species) but even the normally infectious 45S species of Ets-4 RNA had a lower specific infectivity than that of EEE, which indicates that it was biologically defective.

By comparing the patterns of viral RNA species produced by Ets-4 and its parent, one could reasonably state that the control of viral RNA synthesis of Ets-4 was defective. An attempt was then made to understand the control mechanism as a working hypothesis. It was assumed that a regulatory mechanism of EEE operated through a repressor that was absent or defective in Ets-4 - infected cells. This resulted in the production of large amounts of viral RNA that was observed in the mutant. EEE and VEE viruses but not other related and unrelated ones could apparently restore control of RNA synthesis in doubly infected cells to patterns closely resembling those of EEE. Superinfection by EEE had to occur early to be effective (1 hour but not 2 hours), suggesting the possibility that control was determined by an early event following superinfection with EEE virus. "Typical" viral interference was ruled out as an explanation for the restoration of control that was observed, on the basis that none

was demonstrable as judged by the titer of Ets-4 on EEE in singly or doubly infected cells. Furthermore, it was previously shown that interference among group A arboviruses could only be demonstrated if one virus had an advantage in time or in multiplicity of infection over the (inhibited) challenge virus.¹⁸ This was apparently not the case here. In addition, NDV, VSV, and two arboviruses tested that show a potential for interference if they are given an advantage^{19*} failed to restore control of Ets-4 under conditions of simultaneous infection and high multiplicities. One possible explanation of the presumed viral RNA regulatory mechanism is that a viral protein that is common to some of the serologically related group A arboviruses is the repressor that is absent or defective in Ets-4. This is consistent with the inhibition of viral protein synthesis observed in Ets-4 infected cells, and is consistent with other known systems involving protein repressors and viruses.¹⁹⁻²¹ In the case of Ets-4 and EEE, one may speculate further that the viral protein repressor may be a structural protein of the virus, as exemplified by the combined results of Eggen and Nathans¹⁹ and Robertson, Webster, and Zinder.²² They have suggested that control of a viral RNA genome function by the phage coat protein on the synthesis of other phage proteins is involved in an RNA phage infection (translation, according to the Nathans group, and transcription according to the Zinder group). We are considering a somewhat analogous hypothesis that focuses directly on viral RNA synthesis.

Other hypotheses that may account for the apparent loss of control of viral RNA synthesis include (i) a defective feedback inhibition or, alternatively, an end product repression mechanism; and (ii) an explanation based on the fact that the defect in viral protein synthesis permits the Ets-4 RNA to accumulate because the latter cannot be removed from the system (the infected cell) as mature virus. An in vitro system is now under development to test the hypotheses mentioned as well as to define further the parameters and mechanism for the control of viral RNA synthesis in these viruses.

* Unpublished experiments of Zebrovitz and Brown.

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13. ABSTRACT		
<p>The pattern of viral RNA synthesis and growth of a temperature-sensitive mutant (Ets-4) of eastern equine encephalitis (EEE) virus was compared with that of the parent. Growth of Ets-4 was moderately inhibited at 30 and 37 C compared with that of EEE, and was strongly inhibited at 42 C. In Ets-4, similar patterns of inhibitions were observed in the production of complement-fixing antigen and hemagglutinin (HA), which indicated that Ets-4 was defective in viral protein synthesis.</p> <p>The viral RNA synthesis induced by both viruses in infected cells was studied extensively. The mutant produced three times as much total viral RNA as EEE virus at 37 C, but less infectious RNA (IRNA). Comparison of the specific infectivity (IRNA/total viral RNA) of the two viruses showed that of Ets-4 to be 0.1 that of the parent. RNA from each of the viruses was analyzed more completely with sucrose gradient centrifugation. Ets-4 at 37 C produced greater amounts of a noninfectious 20S species (double-stranded forms, ribonuclease-resistant) and a poorly infectious 27S form (single-stranded, ribonuclease-sensitive) of viral RNA. Production of single-stranded infectious species, 45S, of viral RNA by Ets-4 was less than that by EEE virus, but the total uptake of radioactive uridine was higher, suggesting that the 45S species of RNA synthesized in Ets-4 - infected cells was biologically defective.</p> <p>On the basis of the facts noted above, it appeared that viral RNA synthesis in Ets-4 - infected cells was out of control. Control appeared to be restored to the levels and pattern of the parent by superinfection with the parent EEE virus or VEE virus but not with others.</p>		

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